STUDIES IN ACCELERATED AMINO ACID ANALYSIS

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Methods for decreasing the time required for amino acid analysis by ion-exchange chromatography have in general employed smaller and smaller resin particles and faster and faster buffer flow rates through the resin column. These methods have resulted in analysis time reductions from the original 7-day, 2-column method of Moore and Stein (1951) to 1-day (Spackman, Stein, and Moore 1958), to 6-hours (Spackman 1963), and finally to 4-hours (Benson and Patterson 1964; Spackman 1964) for the analysis of the amino acids found in most protein hydrolysates.

The purpose of this report is to define methodology that reduces the time for this type of analysis to slightly more than 2 hours, with flow rates and resins now in common use.

Apparatus and Methods -- A Beckman/Spinco Model 120 B Amino Acid Analyzer, with 2 modifications, was used for this work. First, an accelerated (70 ml/hr) regeneration and equilibration system, shown in Fig. 1, was developed.

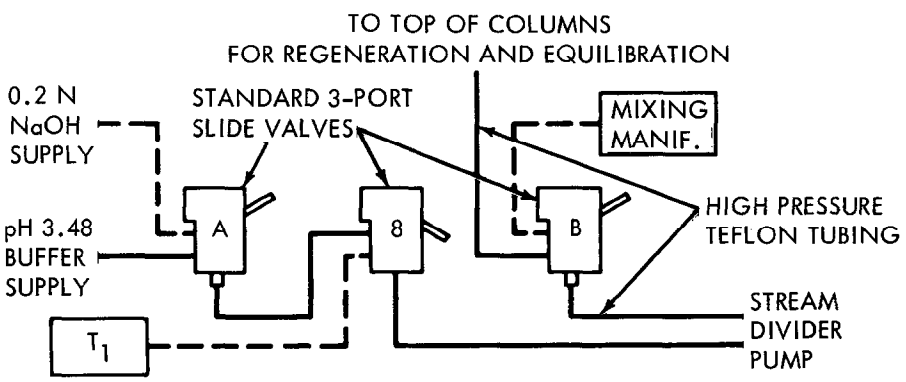

Fig. 1. Accelerated regeneration and equilibration system. Valves shown in equilibration settings.

Two additional standard 3-port valves (A and B) were connected in the system to allow the stream-divider pump to provide flow to the top of the columns for either the 0.2 N NaOH or the pH 3.48 buffer. Stream-division runs can still be performed.

Second, the high-sensitivity cuvettes of Hubbard and Kremen (1964) were used in the Analyzer colorimeter.

The sodium citrate buffers developed by Moore and Stein (1951) and Spackman, Stein, and Moore (1958) were modified as shown in Table 1. No change in the Brij-35 or caprylic acid content was made from the latter reference. Thiodiglycol was used at 5 ml/liter in the sample buffer (pH 2.2), the equilibration buffer (same as buffer 1), and buffers 1 and 2 of Table 1.

Table 1

Modified Sodium Citrate Buffers for Accelerated Chromatography

Buffer	Amino Acids Eluted	pH	N (Na)	Propanol[2] % v/v	Benzyl Alcohol[2] % v/v
1	acidic	3.50 ± .02	0.20	---	---
2	neutral	4.14 ± .02	0.20	3.0	2.3
3	basic	5.18 ± .02	0.35	5.0	---

Two 0.9 x 52 cm columns of PA-28 resin[1] were used for the fractionation of the acidic and neutral amino acids, using buffers 1 and 2 of Table 1, one column being regenerated and equilibrated while the other was running. The basic amino acids were separated on a 0.9 x 4 cm column of PA-35 resin[1], using buffer 3 of Table 1. Flow rates of 70 ml/hr for all buffers and 35 ml/hr for ninhydrin were employed. Back pressures were 70 psi for the short column and 350 psi for the long column.

Operation programs for the Analyzer are indicated on a time scale directly below each of the 2 chromatograms shown (Figs. 2 and 3). The

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[2]J. T. Baker Chemical Co., Phillipsburg, New Jersey

baseline is first set by recording with ninhydrin and buffer 1 effluent.

Results and Discussion -- A chromatogram obtained by using this method with an acid hydrolysate of 0.10 mg of recrystallized ribonuclease is shown in Fig. 2. Actual running time for the chromatogram is 2 hours, 7 minutes, but the next run can be started at the end of 2 hours, before the first sample is finished. The 2-hour period for a run thus allows chromatograms to be started at 8, 10, 12, 2, and 4 o'clock to allow 5 hydrolysate analyses in the normal working day.

Amino acid recoveries at 0.25, 0.15, 0.10, and 0.025 μmole of purified amino acid mixtures are shown in Table 2. The 0.10 μmole levels represent the calibration runs.

Table 2

Recoveries of Amino Acids at Sample Levels from 0.25 to 0.025 μMole

	(Calibration Runs)							Average[a]
μMole Levels		0.10		0.25	0.15	0.05	0.025	± S.D.
Amino Acids:	%	%	%	%	%	%	%	
Lysine	101	102	98	110[b]	99	99	102	100 ± 1.8
Histidine	100	102	98	110[b]	100	103	99	100 ± 1.9
Arginine	101	101	99	105[b]	100	101	100	100 ± 0.8
Aspartic Acid	98	102	100	100	100	99	101	100 ± 1.3
Threonine	98	101	101	103	102	100	101	101 ± 1.6
Serine	98	103	100	99	100	96	101	100 ± 2.2
Glutamic Acid	97	102	101	100	99	99	101	100 ± 1.7
Proline	99	100	100	99	100	98	101	100 ± 1.0
Cystine (Half)	100	100	101	100	99	97	99	99 ± 1.3
Glycine	100	101	99	99	100	97	102	100 ± 1.6
Alanine	98	101	101	100	100	101	102	100 ± 1.3
Valine	99	100	101	100	98	97	100	99 ± 1.4
Methionine	101	100	99	102	101	100	103	101 ± 1.3
Isoleucine	100	100	100	102	101	99	102	101 ± 1.1
Leucine	100	100	100	99	100	99	103	100 ± 1.3
Tyrosine	100	100	100	99	99	100	101	100 ± 0.7
Phenylalanine	100	100	101	100	100	98	102	100 ± 1.2
Average[c]	99	101	100	100	100	99	101	
S.D.	1.2	1.0	1.0	1.3	0.9	1.7	1.2	
2 S.D.	2.4	2.0	2.0	2.6	1.8	3.4	2.4	

[a]Recoveries of each amino acid from 7 runs.
[b]These values omitted from calculations. See text for details.
[c]Recoveries of all amino acids in each run.

The recoveries at the 0.25 μmole sample level in the series of runs shown in Table 2 are very high for the basic amino acids lysine, histidine, and arginine. The first run from a later series, at this

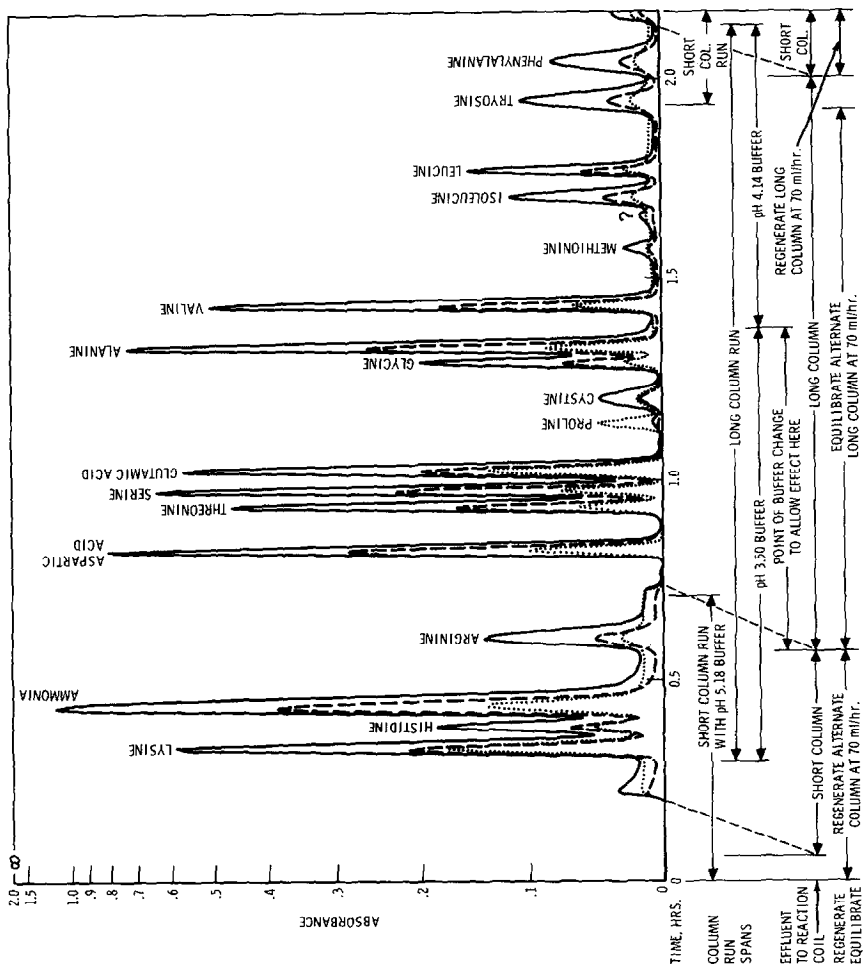

Fig. 2. Chromatogram of 0.1 mg of ribonuclease hydrolysate. Programming of run spans, buffer changes, points for directing effluent to the reaction coil, and a regeneration-equilibration cycle are shown. The unidentified peak between methionine and isoleucine appears to be a peptide and not alloisoleucine.

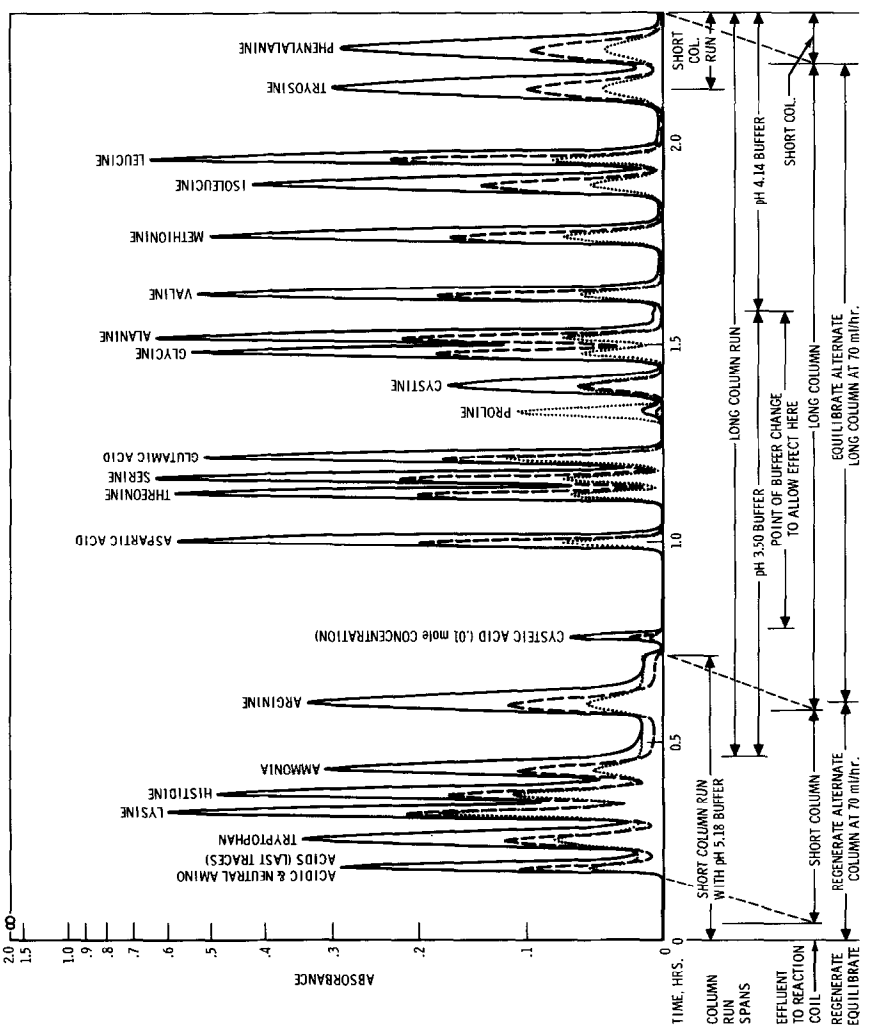

Fig. 3. Chromatogram of a purified amino acid mixture, all at 0.10 μmole except cysteic acid. Programming is shown as in Fig. 2.

same sample level, gave recoveries of 104, 102, and 102 % respectively for these basic amino acids.

Compounds such as cysteic acid in a sample would require that the overlap from one run to the next be decreased, and therefore running time increased. A chromatographic record of a purified amino acid mixture containing cysteic acid and tryptophan is shown with proper programming in Fig. 3. Such a change in programming would allow only 4 samples to be run in the usual working day.

As can be seen on the 2 chromatograms (Figs. 2 and 3), the higher pH of buffer 1 used in this method moves cystine between proline and glycine. This changed sequence not only fills an unused chromatographic space, but also increases the sharpness of the cystine peak. In order to keep the buffer-change peak from appearing under valine (which causes poor recoveries of valine), the pH of buffer 2 had to be decreased. This made the tyrosine-phenylalanine resolution unacceptable. Adding benzyl alcohol, as suggested by the work of Moore and Stein (1951) restored the lost resolution, but the chromatographic peaks were not sharply eluted.

The use of propanol to increase the sharpness of the neutral amino acid peaks was considered because of the work in starch column chromatography by Moore and Stein (1949), and also because of the high boiling point and water miscibility of propanol. The propanol in buffer 3 also increases peak sharpness of the basic amino acids, and moves arginine closer to ammonia and tryptophan forward, away from lysine. Further increases in propanol accentuate this peak movement as the concentration is increased to the 10% level.

Use of propanol and citric acid buffer mixtures over the past 6 months has shown no indication of buffer-storage problems or of ion-exchange column degradation in the Analyzer for periods as great as 3 months.

The use of methanol in citrate buffers, as reported by Thomson and Miles (1964) was also studied in this work. No improvements in resolution were observed, and in general, a retardation of peak elution was found, which became worse as the methanol content was increased from the 5 to the 10% level. Furthermore, the low boiling point of methanol produced gas bubbles in the Analyzer reaction coil, with a consequent discontinuity of flow.

The 350 psi back pressure mentioned earlier for the long column represents a significant disadvantage. One possible solution is the use of a larger resin-particle size. With this some sacrifice in resolution can be expected, but preliminary studies indicate that this is feasible with the AA type Beckman/Spinco resins.

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